(19) Hepatitis

Indispensable Role for TNF-α and IFN-γ at the Effector Phase of Liver Injury Mediated by Th1 Cells Specific to Hepatitis B Virus Surface Antigen[1]

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We report the development and characterization of a novel model of severe hepatitis induced against hepatitis B virus surface Ag (HBsAg). HBsAg was successfully targeted into the liver in soluble form. Using this unique property of HBsAg, we established a liver injury model induced by HBsAg-specific Th1 cells. Severe liver injury was induced in C57BL/6 mice by injection of HBsAg together with HBsAg-specific Th1 cells. Histochemical examination demonstrated extensive necroinflammatory hepatic lesions in these animals. Application of this liver injury model to mutant or gene knockout mice enabled us to define the effector mechanisms of Th1 cells in fulminant hepatitis. When Fas-deficient *lpr* mice were used as recipients, a similar degree of liver injury was induced as in wild-type mice. Moreover, HBsAg-specific Th1 cells obtained from perforin$^{-/-}$ mice could induce severe liver injury in both wild-type and *lpr* mice. These results indicated that neither Fas ligand nor perforin are essential for Th1-mediated liver injury in this model. Pretreatment with anti-TNF-α mAb prevented liver injury, whereas severe liver injury was induced in TNF-α$^{-/-}$ mice. Moreover, IFN-γ receptor-deficient mice were resistant to Th1-mediated liver injury. Therefore, TNF-α and IFN-γ, which were produced by HBsAg-specific Th1 cells during the effector phase, appeared to be indispensable in the pathogenesis of fulminant hepatitis. *The Journal of Immunology,* **2000, 165: 956–961.**

Viral hepatitis has been recognized as an inflammatory condition elicited by T cell-mediated cellular immunity. It has been suggested that the pathogenesis of hepatitis is caused by immune responses to infected cells presenting viral Ag. In turn, these immune responses are beneficial for elimination of virus (1, 2). There is growing evidence suggesting that pathogenic T cell responses are accompanied by activation of Th1-type cellular immunity. Among patients with hepatitis B or hepatitis C infection, those who cleared virus had higher serum IL-12 levels than chronic virus carriers (3), and their CD4$^+$ T cells preferentially produced IFN-γ and IL-2 in response to viral Ags (4, 5). In contrast, chronic hepatitis patients showed weak CTL activity and lacked Th1 responses against viral Ags (6, 7).

Consistent with the observations in human hepatitis, the role of Th1 responses in the pathogenesis of hepatitis was also demonstrated in several experimental liver injury models. In our prior work, we have proposed an important role for IFN-γ-producing Ag-specific CD4$^+$ Th1 cells in the pathogenesis of liver injury (8). In the absence of CD4$^+$ T cells or IFN-γ, liver injury was not invoked by Con A administration (9, 10) or treatment with *Propionibacterium acnes* plus LPS (8, 11). We have also shown a pivotal role for IL-12, which is critically important for the activation of Th1-type immunity, in liver injury elicited by *P. acnes* plus LPS (8). Recently, we established a novel Ag-specific Th1 cell-dependent liver injury model in the absence of nonspecific immune activators such as LPS and Con A (12). The model utilized OVA-specific Th1/Th2 cells induced from TCR-transgenic mice and OVA-containing liposomes to target the Ag toward the liver. Using this model, we demonstrated directly that Th1 cells, but not Th2 cells, could initiate liver injury (12).

The pathogenic mechanism of viral hepatitis has been investigated in hepatitis B virus transgenic murine models (13–15). Chronic liver injury was also demonstrated in hepatitis B virus surface Ag (HBsAg)[3]-transgenic mice, and this model revealed the relevance of prolonged immunological liver damage to hepatocarcinogenesis (16). Acute liver injury was also demonstrated in HBV-transgenic mice by the transfer of HBsAg-specific CTL (14) or Th1 cells (17). For both CTL and Th1 cells, IFN-γ was an essential cytokine for induction of liver injury.

In this report, we document a novel and simple method for eliciting HBsAg-specific liver injury induced by Th1 cells. First, we showed that i.v. injection of small HBsAg protein (24 kDa) resulted in specific accumulation in the liver. Utilizing this unique property of HBsAg, we examined whether adoptive transfer of HBsAg-specific Th1 cells to HBsAg-injected mice could induce liver inflammation. This protocol resulted in acute liver injury, which was transient but more severe than the disease observed in the liver injury induced with OVA-specific Th1 cells (12). We applied this liver injury model to mutant and gene knockout mice,

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[3] Abbreviations used in this paper: HBsAg, hepatitis B virus surface Ag; IFN-γR, IFN-γ receptor; iNOS, inducible NO synthase.

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including Fas-deficient (*lpr*), perforin$^{-/-}$, TNF-$\alpha^{-/-}$, and IFN-γ receptor$^{-/-}$ (IFN-γR$^{-/-}$) mice, to evaluate the molecular mechanisms underlying Th1 cell-mediated liver injury. We demonstrate a requirement for TNF-α and IFN-γ produced by HBsAg-specific Th1 cells during the effector phase of liver injury.

Materials and Methods

Mice

C57BL/6J mice and C57BL/6J-*lpr* mice were obtained from Japan SLC (Shizuoka, Japan) and used at 6–8 wk of age. Perforin$^{-/-}$-(C57BL/6 × 129SvEv) mice were obtained from Taconic (Germantown, NY). TNF-$\alpha^{-/-}$-C57BL/6 mice were provided by Dr. K. Sekikawa (Department of Immunology, National Institute of Animal Health, Tsukuba, Japan). IFN-γR$^{-/-}$-C57BL/6 mice were provided by Dr. Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

Reagents and mAbs

Recombinant yeast-derived small HBsAg (>99% pure) was donated by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). IL-12 was donated by Genetics Institute (Cambridge, MA). Anti-IL-4 mAb (11B11) was purchased from American Type Culture Collection (Manassas, VA). Recombinant mouse IFN-γ and anti-IFN-γ mAb (R4-6A2) were purchased from PharMingen (San Diego, CA). Anti-TNF-α mAb (MP6-XT22) was a gift from Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan). Freeze-dried liposomes (Coatsome EL-A-01) were kindly donated by NOF (Tokyo, Japan), and OVA-containing liposomes were prepared as described previously (12).

Preparation of HBsAg-specific Th1 cells

HBsAg (100 μg/mouse) emulsified in CFA (Difco, Detroit, MI) was injected into female C57BL/6 mice. After 2 wk, mice were further immunized with HBsAg emulsion in IFA (Difco), and this treatment was repeated four times at 2-wk intervals. Spleen cells from the immunized mice were stimulated with 20 μg/ml HBsAg in the presence of 20 U/ml IL-12, 1 ng/ml IFN-γ, 50 μg/ml anti-IL-4 mAb, and 20 U/ml IL-2. After 72 h, CD8$^+$ T cells in this culture were removed using Dynabeads (Dynal AS, Oslo, Norway), and the CD8$^+$ T cell-depleted fraction was restimulated under the conditions described above with mitomycin C-treated C57BL/6 spleen cells. Then, Th1 cells were expanded in the presence of high concentrations of IL-2 (80 U/ml). Cytokine levels (IFN-γ and IL-4) produced by these cells were determined by ELISA (PharMingen). HBsAg-specific Th1 cells from perforin$^{-/-}$ mice were obtained by the same method.

Detection of HBsAg

FITC-conjugated HBsAg (FITC-HBsAg) was prepared as described previously (12). C57BL/6 mice were injected i.v. with 15 μg FITC-HBsAg/0.2 ml PBS. Tissue samples were obtained after 2 h, fixed in 1% glutaraldehyde/4% paraformaldehyde/PBS for 6 h, and frozen in liquid N_2 using OCT compound (Sakura Finetechnical, Tokyo, Japan). Tissue blocks were sectioned and examined by fluorescence microscopy.

Induction of liver injury

C57BL/6 mice were treated first with i.v. injection of HBsAg (in 200 μl saline) and 2 h later with cell transfer. Cultured HBsAg-specific Th1 cells were washed and resuspended in saline, and 2 × 10^7 cells (in 200 μl saline) were injected i.v. at a volume of 200 μl. Mice were sacrificed after 24 h unless stated otherwise and sera were collected to determine aspartate aminotransferase and alanine aminotransferase concentrations as described previously (8). Sera were also tested for IFN-γ levels by ELISA (PharMingen). Tissue samples were fixed in 10% formalin-PBS and embedded in paraffin, and sections were stained with hematoxylin and eosin. In some experiments, mice were pretreated i.p. with 500 μg/mouse of anti-IFN-γ mAb or anti-TNF-α mAb 24, 1 h before the injection of HBsAg.

Results

Deposition of i.v. injected soluble HBsAg protein into the liver

To investigate disposition of i.v.-injected HBsAg, we administered 15 μg FITC-HBsAg per mouse to C57BL/6 mice through the tail vein and examined distribution of HBsAg by fluorescence microscopy (Fig. 1). After 2 h, fluorescence was detected in the liver (Fig. 1, *A* and *B*) and spleen (Fig. 1*C*) but was barely found in the kidney (Fig. 1*D*). As shown in Fig. 1*A*, FITC-HBsAg was equally distributed across the liver lobule. Fluorescence was present along the sinusoid lining cells but was not found in parenchymal cells (Fig. 1*B*). In the spleen, FITC-HBsAg was located mainly in the marginal zone and red pulp (Fig. 1*C*). We could not observe such a specific distribution by injection of FITC-labeled OVA protein Ag (data not shown), indicating that this tissue distribution was specific to HBsAg.

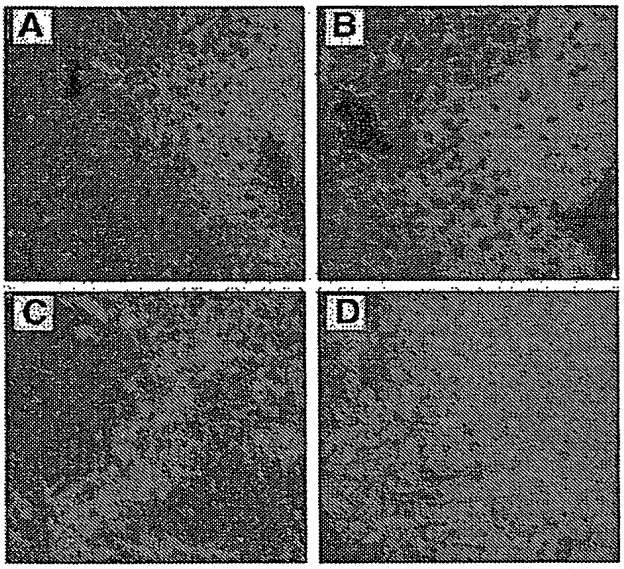

FIGURE 1. Fluorescence micrographs of tissue distribution of FITC-HBsAg. C57BL/6 mice were i.v. injected with FITC-HBsAg (15 μg/mouse), and tissue samples were collected after 2 h. Frozen sections from the liver (*A, B*), spleen (*C*), and kidney (*D*) were examined by fluorescence microscopy as described in *Materials and Methods*.

Establishment of a liver injury model induced by HBsAg-specific Th1 cells

We have previously demonstrated liver injury could be induced against OVA proteins targeted to the liver with liposomes by adoptive transfer of OVA-specific Th1 cells (12). We therefore tested whether adoptive transfer of HBsAg-specific Th1 cells to HBsAg-treated mice could induce hepatitis. HBsAg-specific Th1 cells were prepared from mice immunized with HBsAg by repeated restimulation in vitro in the presence of Th1-biasing cytokines. The resulting cell population included >99% CD4$^+$ T cells and produced IFN-γ, but no IL-4, on restimulation with HBsAg (data not shown). When C57BL/6 mice were injected with these Th1 cells after i.v. administration of HBsAg, a marked elevation of serum transaminase levels was noted, whereas HBsAg or Th1 cells alone had no effect (Fig. 2). Furthermore, HBsAg-specific Th1 cells did not induce liver injury in combination with OVA, targeted to the liver using liposomes. Therefore, Th1 cells induced liver injury in an Ag-specific manner. Moreover, no hepatic injury was observed even when OVA-specific Th1 cells were injected into mice after i.v. injection of soluble OVA (data not shown). Therefore, HBsAg, combined with HBsAg-specific Th1 cells, appeared to be unique in its ability to induce liver injury.

Histochemical examination also demonstrated strong liver damage in mice treated with HBsAg and Th1 cells. In contrast with normal tissue (Fig. 3*A*), there were necroinflammatory foci with degenerating hepatocytes and hemorrhage (Fig. 3*D*). Such highly inflammatory areas were prevalent in the liver and frequent at the

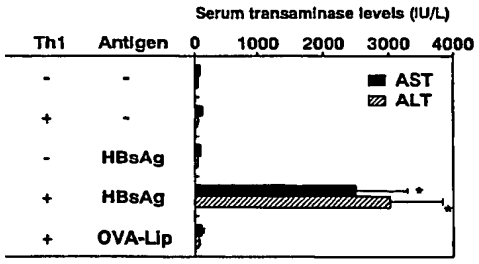

FIGURE 2. Induction of liver injury by combined treatment with HBsAg and HBsAg-specific Th1 cells. Mice were treated with HBsAg (15 μg) and/or Th1 cells (2×10^7 cells); 24 h later, serum transaminase levels were examined. As an alternative Ag, OVA (160 μg)-containing liposomes (OVA-Lip) were i.v. injected instead of HBsAg. Data are means ± SE of five mice. Statistical significance was calculated by Student's *t* test. *, $p < 0.05$ vs control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

edge of the tissue, and we could observe necrotic white spots macroscopically on the surface of the liver (data not shown). Treatment with HBsAg and Th1 cells also induced histopathological changes in the spleen (Fig. 3*E*). However, we could not observe any changes in the kidney (Fig. 3*F*). Thus, it appeared that HBsAg-specific Th1 cell-mediated tissue injury occurred at those sites where i.v. injected HBsAg accumulated (Fig. 1).

In addition to its Ag specificity, the severity of Th1 cell-induced liver injury was dependent on the dose of HBsAg. At a dose of 7.5 μg/mouse, two of four mice showed strong hepatic injury but the rest were minimally affected. A dose of 15 μg/mouse was sufficient for optimal liver injury in all mice, and no further increase was observed even when doses were as high as 60 μg/mouse (Fig. 4*A*). As for cell number, adoptive transfer of 1×10^7 Th1 cells to the mice could induce liver injury as strong as 2×10^7 Th1 cells and 5×10^6 Th1 cells was also pathogenic with diminished liver damage (data not shown). However, cell numbers lower than 2×10^7 are not always pathogenic to all mice in the same group, and we used 2×10^7 cells/mouse in the following experiments. To characterize the duration of this liver injury, we examined kinetics of liver injury in mice treated with 15 μg HBsAg and 2×10^7 Th1 cells. Increase of serum transaminase levels became prominent at 16 h and reached a peak at 24 h (Fig. 4*B*). The extent of liver injury

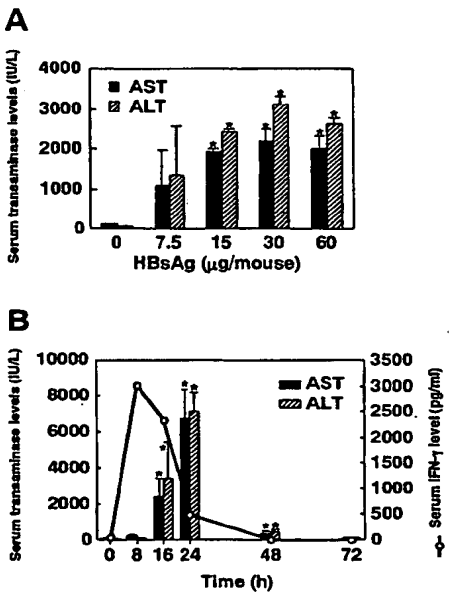

FIGURE 4. Dose dependency and kinetics of liver injury induced by HBsAg-specific Th1 cells. *A*, Induction of liver injury by Th1 cells in response to increasing doses of HBsAg. Mice were injected with the indicated doses of HBsAg 2 h before the transfer of Th1 cells (2×10^7 cells). Transaminase levels in the sera were determined after 24 h as described in *Materials and Methods*. Data are means ± SE of four mice. *B*, Transient liver injury by HBsAg-specific Th1 cells. Mice were treated with HBsAg (15 μg) and Th1 cells (2×10^7 cells) at a 2-h interval and sacrificed at the indicated times. Sera were examined for transaminase levels and IFN-γ levels as described in *Materials and Methods*. Data are means ± SE of five mice. Statistical significance was calculated by Student's *t* test. *, $p < 0.05$ vs control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

gradually reduced thereafter rather than persist; after 48 h, serum transaminase levels diminished to 6% of maximum value although these levels were still significantly higher than those of controls and recovered to control levels by 72 h. An elevation of serum IFN-γ levels always preceded the liver injury (Fig. 4*B*). It was maximum 8 h after cell transfer, began to fall after 16 h, and

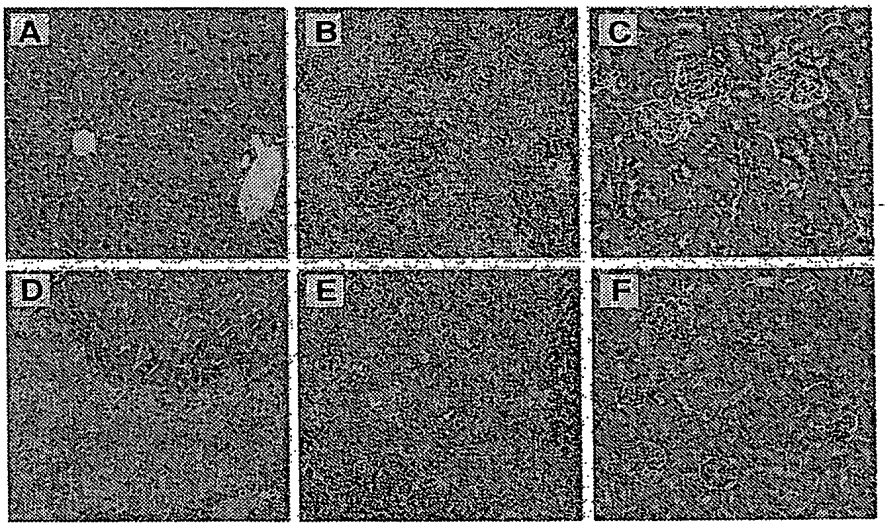

FIGURE 3. Histological examination of HBsAg-specific Th1 cell-dependent liver injury. Mice were treated with HBsAg (15 μg) and Th1 cells (2×10^7 cells) at a 2-h interval. Liver (*D*), spleen (*E*), and kidney (*F*) were obtained after 24 h, fixed in 10% formalin-PBS, and examined by hematoxylin/eosin staining. Liver (*A*), spleen (*B*), and kidney (*C*) from untreated mice are also shown.

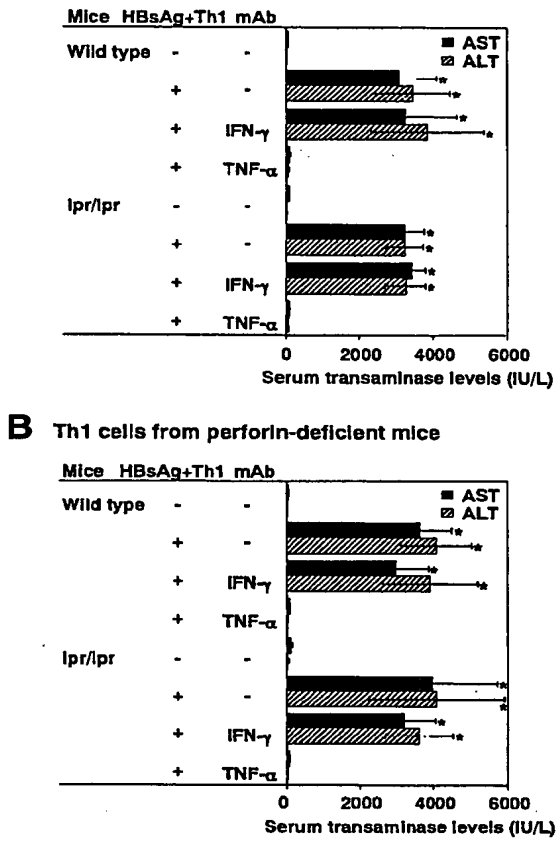

FIGURE 5. Effector mechanisms involved in the pathogenesis of liver injury induced by Th1 cells. Wild-type C57BL/6 mice or Fas-deficient C57BL/6-*lpr* mice were treated with HBsAg (15 μg) and HBsAg-specific Th1 cells (2×10^7 cells) derived from wild-type mice (*A*) or perforin-deficient mice (*B*). Some groups of mice were pretreated with anti-IFN-γ mAb or anti-TNF-α mAb (500 μg/mouse i.p.) 24 and 1 h before injection with HBsAg and Th1 cells. Serum transaminase levels were determined after 24 h. Data are means ± SE of five mice. Statistical significance was calculated by Student's *t* test. *, $p < 0.05$ vs control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

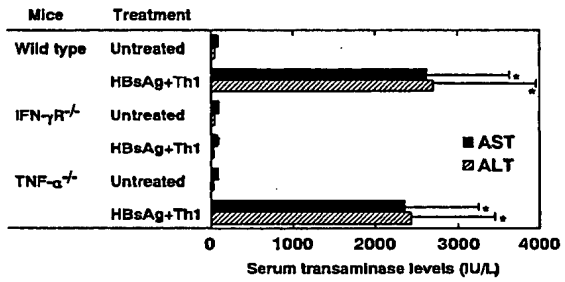

FIGURE 6. Essential role for TNF-α and IFN-γ/IFN-γR signaling pathways in liver injury induced by Th1 cells. Wild-type, IFN-γR$^{-/-}$, or TNF-α$^{-/-}$ mice on a C57BL/6 background were treated with HBsAg (15 μg) and HBsAg-specific Th1 cells (2×10^7 cells) at a 2-h interval. After 24 h, sera were examined for transaminase levels and IFN-γ levels as described in *Materials and Methods*. IFN-γ was not detected in the sera from untreated mice, and serum IFN-γ levels from treated mice were as follows: wild-type mice, 5551 ± 1604 pg/ml; IFN-γR$^{-/-}$ mice, 6722 ± 2908 pg/ml; TNF-α$^{-/-}$ mice, 5087 ± 1353 pg/ml. Data are means ± SE of five mice. Statistical significance was calculated by Student's *t* test. *, $p < 0.05$ vs control group. AST, aspartate aminotransferase; ALT, alanine aminotransferase.

further declined after 24 h when serum transaminase levels were maximum. These results suggested that passively transferred HBsAg-specific Th1 cells triggered a cascade of liver injury.

Role of Fas ligand and perforin in the liver injury induced by HBsAg-specific Th1 cells

We next examined the mechanism by which Ag-specific Th1 cells cause severe liver injury. One explanation may be the direct cytolytic action of Th1 cells via Fas ligand or perforin. However, when Fas-deficient *lpr* mice were used as recipients, the extent of liver injury was equal to that of wild-type mice (Fig. 5*A*). This suggested that lack of Fas-Fas ligand interaction did not inhibit liver injury. Furthermore, we induced HBsAg-specific Th1 cells from perforin$^{-/-}$ mice to investigate the precise role of perforin in Th1-mediated liver injury. As clearly shown in Fig. 5*B*, these perforin-deficient HBsAg-specific Th1 cells induced liver injury that was as severe as the injury induced by wild-type Th1 cells. Perforin-deficient Th1 cells could also induce severe liver injury in *lpr* mice (Fig. 5*B*). From these results, we concluded that neither Fas ligand nor perforin were required for the effector function of Th1 cells in this model for liver injury.

Requirement of Th1-derived TNF-α and IFN-γ in the liver injury induced by HBsAg-specific Th1 cells

The results described above showing that direct cytolytic action of Th1 cells was not responsible for the liver injury prompted us to test the participation of cytokines in liver injury. As shown in Fig. 5, we examined effects of anti-IFN-γ mAb and anti-TNF-α mAb in wild-type mice and *lpr* mice given Th1 cells from wild-type mice or perforin$^{-/-}$ mice. In all combinations of recipient mice and Th1 cells, pretreatment with anti-TNF-α mAb suppressed the levels of serum transaminase to control levels. On the other hand, TNF-α$^{-/-}$ mice revealed severe liver injury on treatment with HBsAg and Th1 cells (Fig. 6). These results suggest that TNF-α produced by Th1 cells is a very important factor in the pathogenesis of liver injury.

IFN-γ has been shown to be a crucial factor in several hepatitis models (10, 17, 18). We have also observed a protective effect of anti-IFN-γ mAb in *P. acnes* plus LPS-induced hepatitis (8) and in OVA-specific Th1 cell-dependent liver injury (12); however, anti-IFN-γ mAb did not block liver injury in this model (Fig. 5). Suppression of serum IFN-γ level was verified by ELISA, which indicated that the level was under the limit of detection. Failure of anti-IFN-γ mAb to block the liver injury can be interpreted as follows: 1) IFN-γ is not responsible for the pathogenesis of liver injury; or 2) the very small amounts of IFN-γ that escaped neutralization by anti-IFN-γ mAb were sufficient to cause liver injury. To distinguish between these alternative explanations, we examined IFN-γR$^{-/-}$ mice as recipients. As shown in Fig. 6, IFN-γR$^{-/-}$ mice showed no symptom of liver injury on treatment with HBsAg and Th1 cells. Thus, we concluded that IFN-γ is indispensable for the induction of liver injury and that small amounts of IFN-γ are sufficient.

Discussion

We have previously shown that IFN-γ-producing CD4$^+$ T cells play a critical role in the pathogenesis of liver injury induced by *P. acnes* plus LPS (8, 11). In addition, pretreatment with anti-IL-12

mAb completely suppressed *P. acnes* plus LPS-induced liver injury (8). Therefore, Th1-type immunity during the priming phase appeared to be essential to *P. acnes*-induced injury; however, it was also reported that IL-4-producing Th2-type $CD4^+$ T cells contribute to the effector phase after LPS administration (19). Recently, we have established an Ag-specific $CD4^+$ T cell-dependent liver injury model using a combination of OVA-specific Th cells and OVA-containing liposomes (12). Using this model, we demonstrated that Ag-specific Th1 cells, but not Th2 cells, are responsible for the onset of liver injury. Here we established a liver injury model induced by HBsAg-specific Th1 cells and showed a critical role of IFN-γ and TNF-α in the pathogenesis of the resulting liver injury.

In a previous report, we described a liver injury model induced by OVA-specific Th1 cells and OVA-containing liposomes (12). Liposomes are useful for carrying compounds into the liver. Here, however, we found that the small HBsAg protein did not require liposomal encapsulation for targeting into the liver. Although its mechanism for uptake is unclear, intact small HBsAg protein distributed to the liver and spleen but not to the kidney (Fig. 1). HBsAg, the envelope protein of the virus, has been studied to clarify how the virus attaches and penetrates to target cells (20). To date, several cell surface molecules have been identified as HBsAg-binding proteins. Among them, apolipoprotein H (21, 22) and annexin V (23, 24) are candidates for the small HBsAg attachment site. It is possible that these molecules expressed in the liver and spleen may contribute to the interaction with small HBsAg protein, although other mechanisms such as endocytosis by phagocytes are also possible. In any case, tissue damage corresponded with the tissue distribution of injected HBsAg (Figs. 1 and 3). These results suggest that APCs can process HBsAg and present antigenic peptide on their surface MHC class II molecules, which leads to activation of immune responses elicited by Th1 cells and eventually tissue injury.

We have established two Ag-specific Th1 cell-inducible liver injury models using the same strategy, OVA-specific model and HBsAg-specific model, yet the magnitude of liver injury induced was different. Moderate liver damage occurred in the OVA-specific model (12), whereas severe injury was induced in the HBsAg model as shown by 10-fold higher elevation of serum transaminase levels. Although these two models utilize a similar method, there are some differences that may affect the magnitude of liver injury. One possible explanation is that the availability of the antigenic epitopes by HBsAg-specific Th1 cells may be greater than that by OVA-specific Th1 cells. Because OVA-specific Th1 cells were induced from $OVA_{323-339}$-specific TCR-transgenic mice, these cells can recognize only the $OVA_{323-339}$ epitope among the antigenic epitopes of OVA presented by MHC class II molecules. On the other hand, we induced HBsAg-specific Th1 cells from mice immunized with HBsAg, and it is expected that this polyclonal population recognizes multiple antigenic epitopes of HBsAg. This difference may result in a larger dose of antigenic HBsAg peptides that can activate Th1 cells and cause more severe tissue damage.

Another possible factor is the genetically controlled difference of Th1/Th2 balance in mice. The OVA-specific model and HBsAg-specific model were established on the BALB/c and C57BL/6 backgrounds, respectively. As we have shown previously, C57BL/6 mice are more susceptible than BALB/c mice to *P. acnes* plus LPS-induced liver injury (8). Similar strain differences were reported for the Con A-liver injury model (25). Conversely, BALB/c mice are susceptible to *Leishmania major* infection whereas C57BL/6 mice are resistant (26–28). Furthermore, when stimulated with anti-CD3 mAb, $CD4^+$ T cells from C57BL/6 mice produced significantly lower levels of IL-4 than $CD4^+$ T cells from BALB/c mice (29). These results imply effective activation of Th1-type immune responses in C57BL/6 mice so that these animals can eliminate intracellular pathogens, yet they are susceptible to severe liver injury. Although BALB/c mouse-derived Th1 cells produce equal or even higher levels of IFN-γ than C57BL/6 mouse-derived Th1 cells in our liver injury models, the magnitude of liver injury on the BALB/c background is much lower than that of C57BL/6 background. Therefore, it is also possible that the progression of Th1-type immune responses triggered by IFN-γ is suppressed in BALB/c mice compared with C57BL/6 mice.

Recent findings suggest a central role of $CD4^+$ T cells in antitumor responses and $CD4^+$ T cell-mediated activation of effector cells, including $CD8^+$ T cells, eosinophils, and macrophages (30, 31). As for liver injury models, involvement of $CD8^+$ CTLs (14) and NKT cells (32) was also reported. Although direct interaction is uncertain, HBsAg-specific Th1 cells may activate the recipient's effector cells to increase the liver injury. Consistent with this idea, Yoneyama et al. (19) demonstrated that Th1 responses primed by *P. acnes* were followed by release of thymus and activation-regulated chemokine on LPS administration, recruitment of IL-4-producing $CCR4^+$ $CD4^+$ T cells, and massive liver damage. Likewise, we cannot exclude the possibility that HBsAg-specific Th1 cells and the recipient's immune system may act synergistically to develop the severe tissue damage.

We examined the relative contribution of Fas ligand, perforin, IFN-γ, and TNF-α to the liver injury induced by Ag-specific Th1 cells and showed the requirement for IFN-γ and TNF-α and no requirement for Fas ligand and perforin (Figs. 5 and 6). Similarly, Nakamoto et al. (15) reported that IFN-γ-producing CTL kill hepatocytes without Fas ligand and perforin in an Ag-specific manner. In several experimental hepatitis models established to date, IFN-γ and TNF-α were demonstrated to play an important role in the pathogenesis of liver injury (8, 10, 12, 17, 18, 33–35). In our model, anti-TNF-α mAb strongly blocked Th1 cell-inducible liver injury (Fig. 5), but TNF-$\alpha^{-/-}$ mice were susceptible to liver injury (Fig. 6). Thus, our results suggested the importance of TNF-α production by Th1 cells. As for IFN-γ, although anti-IFN-γ mAb was not effective (Fig. 5), resistance of IFN-$\gamma R^{-/-}$ mice suggested the necessity of IFN-γ to liver injury (Fig. 6). These results suggest requirement of both IFN-γ and TNF-α in this liver injury. It can be postulated that IFN-γ and TNF-α produced by ligand-stimulated Th1 cells prime APC for activation of cytotoxic effector cells. In this mechanism, inducible NO synthase (iNOS) induced by IFN-γ and TNF-α may participate to the cytotoxicity. Induction of iNOS has been reported in human chronic viral hepatitis (36) and mouse liver injury model (37). It is also possible that IFN-γ sensitizes liver cells to the cytotoxicity of TNF-α (38, 39). To elucidate their mechanism of action, the possible synergistic effect of IFN-γ and TNF-α in the induction of liver injury should be further examined.

In conclusion, we established a novel liver injury model induced by HBsAg-specific Th1 cells. The development of severe liver injury in this model will be useful for examination of the mechanisms underlying Ag-specific fulminant hepatitis. Here, we clearly demonstrated that TNF-α and IFN-γ produced by HBsAg-specific Th1 cells are critically important in the late effector phase of acute liver injury.

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